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A study on Nim expression in *Bacteroides fragilis*

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Members of the genus *Bacteroides*, mainly *Bacteroides fragilis*, can cause severe disease in man, especially after intestinal perforation in the course of abdominal surgery. Treatment is based on a small number of antibiotics, including metronidazole, which has proved to be highly reliable throughout the last 40 to 50 years. Nevertheless, metronidazole resistance does occur in *Bacteroides* and has been mainly attributed to Nim proteins, a class of proteins with a suggested nitroreductase function. Despite the potentially high importance of Nim proteins for human health, information on the expression of *nim* genes in *B. fragilis* is still lacking. It was the aim of this study to demonstrate expression of *nim* genes in *B. fragilis* at the protein level and, furthermore, to correlate Nim levels with the magnitude of metronidazole resistance. By the application of 2D gel electrophoresis, Nim proteins could be readily identified in *nim*-positive strains, but their levels were not elevated to a relevant extent after induction of resistance with high doses of metronidazole. Thus, the data herein do not provide evidence for Nim proteins acting as nitroreductases using metronidazole as a substrate, because no correlation between Nim levels and levels of metronidazole resistance could be observed. Furthermore, no evidence was found that Nim proteins protect *B. fragilis* from metronidazole by sequestering the activated antibiotic.

INTRODUCTION

The intestinal bacteria of the genus *Bacteroides* account for as much as 30 %, approximately, of human faecal isolates (Kuwahara *et al.*, 2004) and utilize carbohydrate sources that are inaccessible to the human host. In general, *Bacteroides* spp. are beneficial to the host by producing volatile fatty acids that can be absorbed through the large intestine (Wexler, 2007). However, *Bacteroides* spp., predominantly *Bacteroides fragilis*, can also cause severe disease, especially after abdominal surgery or injury of the gastrointestinal tract (Aldridge & Sanders, 2002). Main symptoms include abscess formation (abdomen, liver, brain, lungs) and/or bacteraemia (Wexler, 2007). Treatment schemes mainly rely on ampicillin/sulbactam, clindamycin, carbapenems and the 5-nitroimidazole drug metronidazole (Wexler, 2007). Presently, carbapenems and metronidazole are the most reliable treatment options because resistance rates have remained very low (Hedberg & Nord, 2003; Sóki *et al.*, 2013). Nevertheless, metronidazole-resistant *Bacteroides* isolates have been repeatedly isolated and seem to be relatively common even in some parts of the world, e.g. in the UK (Brazier *et al.*, 1999). In most cases, metronidazole resistance is associated with the presence of *nim* genes, which were first described as transmissible metronidazole-resistance determinants (Breuil *et al.*, 1989). They can be either chromosomal or plasmid borne, and are often, but not always, associated with insertion elements (Sóki *et al.*, 2006). To date, ten isoforms of *nim* have been described, *nimA–J* (Gal & Brazier, 2004; Husain *et al.*, 2013), but there is currently no evidence that they act differently. In fact, the mode of action of Nim proteins has not been fully elucidated as yet, but they are commonly believed to act as nitroreductases, which reduce the nitroimidazole drug's nitro group to a barely reactive amino group (Carlier *et al.*, 1997). In contrast with this notion, however, only a fraction of *nim*-positive strains are metronidazole resistant (Gal & Brazier, 2004; Löfmark *et al.*, 2005), and the introduction of *nimE* and *nimJ* into *B. fragilis* 638R had only a very modest effect on metronidazole susceptibility (Husain *et al.*, 2013). This discrepancy can possibly be reconciled by the observation that induction of high-level metronidazole resistance is far easier in *nim*-positive *Bacteroides* isolates than in *nim*-negative isolates (Löfmark *et al.*, 2005), suggesting elevated Nim expression in challenged isolates. Evidence for this, however, is presently lacking as no Nim expression studies have been conducted to date.

Abbreviation: 2DE, 2D gel electrophoresis.

One supplementary figure is available with the online version of this paper.

The present study aimed at filling this gap, and thereby constitutes what is to the best of our knowledge a first attempt to correlate Nim levels as such to metronidazole resistance. To this end, several *B. fragilis* isolates carrying a *nim* gene were studied by 2D gel electrophoresis (2DE) in order to quantify abundancies of Nim. Furthermore, it was assessed whether *nim* gene levels are being upregulated in response to exposure to ever increasing concentrations of metronidazole. The data presented here may prove instrumental for a further assessment of the role of *nim* genes in the development of metronidazole resistance in *Bacteroides* spp.

METHODS

Chemicals and growth media components. Metronidazole, tinidazole, haemin, DTT, CHAPS, urea, thiourea, acetone, TCA and bisacrylamide were obtained from Sigma-Aldrich. Brain heart infusion (BHI) broth and vitamin K1 were purchased from Carl Roth. Wilkins–Chalgren (WC) anaerobe agar was purchased from Oxoid. Acrylamide, IPG (immobilized pH gradient) strips for IEF, ampholytes and iodoacetamide were purchased from Bio-Rad. Yeast extract and Anaerocult A for anaerobic culture were obtained from Merck. Coomassie brilliant blue was purchased from Serva.

Bacterial strains and culture. The following *B. fragilis* strains were used for this study: 638R, either without plasmid or with either pIP417 (*nimA*) or pIP421 (*nimD*); BF-8 (*nimB*, chromosomal); and 388/1 (*nimE* on plasmid pBF388C). The *nimA* gene on pIP417 is associated with IS*1186*, whereas *nimD* on pIP421 is associated with IS*1169*. The *nimB* gene in BF-8 had been found to be associated with an IS*1186*-like insertion element (Haggoud *et al.*, 1994), and *nimE* on pBF388C is associated with an IS-like element (IS*Bf6*). Strains BF-8 (Sebald *et al.*, 1990; Sóki *et al.*, 2004, 2006) and 388/1 (Sóki *et al.*, 2004; 2006) had been found to be resistant to 8 mg metronidazole l^{-1} and 16 mg metronidazole l^{-1}, respectively.

Cultures were routinely grown in anaerobic jars on WC plates at 37 °C. When batch cultures were needed for 2DE, bacteria were grown in 25 ml tissue culture flasks (Falcon; Becton Dickinson) in BHI broth supplemented with 0.5 % yeast extract, 5 mg haemin l^{-1} and 1 mg vitamin K l^{-1} (BHI-S), as described by Jousimies-Somer & Summanen (2002). Bacterial culture was carried out in anaerobic jars (Merck) using the Anaerocult A system.

Induction of high-level metronidazole resistance in *B. fragilis* and determination of MIC. High-level resistance was induced by growing strains on a series of WC agar plates with increasing concentrations of metronidazole until a maximal concentration of 256 mg l^{-1} was reached. The concentration of metronidazole in the plates was increased in doubling steps, i.e. 1, 2, 4, 8, 16, 32, 64, 128 and finally 256 mg l^{-1}. The MIC was defined as the lowest metronidazole concentration at which no more *B. fragilis* growth on WC plates could be observed.

Exposure of *B. fragilis* 638R batch cultures to metronidazole. After 16 h, stationary phase cultures (20 ml) were divided equally into two 25 ml culture flasks and 10 ml fresh BHI-S was added to both flasks. Either 50 μM metronidazole or 50 μM tinidazole was added to one of the cultures, followed by 2 h incubation in an anaerobic jar at 37 °C. Afterwards, samples were processed as described below.

Sample preparation for 2DE. *Bacteroides fragilis* cultures (20 ml) were harvested at 3000 ***g*** for 10 min at 4 °C in a Sigma 4K15 centrifuge. The pellet was resuspended and washed in 1 × PBS and repelleted again at 3000 ***g*** for 10 min at 4 °C. Bacteria were then resuspended in a small volume (400 μl) of ultrapure water, and proteins were precipitated by adding 12.5 % TCA in acetone to the sample until a final concentration of 10 % TCA was reached. Proteins were precipitated for at least 1 h at −20 °C. The precipitates were pelleted in a Sigma 1-15PK cryo-centrifuge (20 000 ***g***, 20 min, 4 °C) and pellets were washed in 90 % acetone (20 000 ***g***, 20 min, 4 °C). Pellets were then dried and resuspended in 2DE sample buffer (7 M urea, 2 M thiourea, 4 % CHAPS, 1 % DTT, 1 % ampholytes, pH 3–10) for at least 3 h. Insoluble matter was removed by centrifugation at 20 000 ***g*** for 20 min at 20 °C. The protein concentration in the supernatant was determined by Bradford assay (Bradford, 1976), and 400 μg protein was used for 2DE.

2DE. IEF was performed in 17 cm IPG strips with pH 3–10 nonlinear (Bio-Rad) and a Protean IEF cell (Bio-Rad), using the following program: 12 h rehydration at 50 V, 1 h 150 V (rapid slope), 1 h 300 V (rapid slope), 1 h 2000 V (linear slope), 2 h 5000 V (linear slope) and 7 h 8000 V (rapid slope). After IEF, gel strips were equilibrated in a two-step procedure in 6 M urea, 30 % glycerol, 2 % SDS and 50 mM Tris pH 8.8 containing either 1 % DTT (first step) or 4 % iodoacetamide (second step). Strips were run vertically in a Protean II xi cell (Bio-Rad) at 22 mA overnight (4 °C). After gel electrophoresis, gels were stained with Coomassie brilliant blue R-250 and scanned in an Epson V750 Pro scanner. Image analysis was performed using Melanie 4 software (GeneBio).

In-gel tryptic digestion and mass-spectrometric analysis of isolated proteins. Tryptic digestion of Coomassie-stained protein spots was performed by reversed phase LC ESI-ion trap MS-MS on a Bruker amaZon ETD speed ion trap (Bruker Daltonics) coupled to a Dionex Ultimate 3000 UHPLC system (Dionex–ThermoFisher) as described previously (Kolarich *et al.*, 2012), with minor modifications as given in detail in Fig. S1, available in the online Supplementary Material.

Data analysis was performed with ProteinScape 3 (Bruker Daltonics) and MASCOT 2.3 (MatrixScience) using the following search parameters: cysteine as carbamidomethyl was set as fixed modification, while deamidation (Asn/Gln) and oxidation (Met) were set as variable modifications. A maximum of two missed cleavages were allowed. Peptide tolerance (both MS and MS-MS) was set at ±0.2 Da. The data were searched against the National Center for Biotechnology Information protein database.

Quantitative PCR analysis of the effect of metronidazole on plasmid copy number. Copy numbers of the *nimA* gene in *B. fragilis* 638R/pIP417 were determined by the $\Delta\Delta C_T$ method using SYBR Green detection, the housekeeping gene glyceraldehyde-phosphate dehydrogenase (*gap*) as a reference, and the pIP417 *repA* and *nimA* genes as target genes. Copies of the *nimA* gene in clones of 638R/pIP417 adapted to 2 μg metronidazole ml^{-1} and 256 μg metronidazole ml^{-1} (adaptation to metronidazole having been carried out as described above) were measured using unchallenged *B. fragilis* 638R/pIP417 as the control. Samples were prepared by the boiling method (Sóki *et al.*, 2013) and the primers used were as follows: *gap*, gapd1BF 5′-AGCCATTGTAGCAGCTTTTT-3′ and gapd3BF 5′-GAAAACATCATCCCGTCT-3′; $repA_{417}$, repA417-1 5′-TGAGCAACCAGAAAACTC-3′ and repA417-2 5′-TTTTTGCAGCATCCACAA-3′; and *nimA*, nimART1 5′-GTTCCTGCCGAGTTTACAAC-3′ and nimART2 5′-GATGGTCGAATCCCTTGTCT-3′. The PCRs included 5 μl SYBR Select mastermix (Life Technologies), 0.7 μM primers and 1 μl of template preparations in 10 μl final volumes in triplicate in 48-well PCR plates. Amplifications (95 °C 10 min; 35 cycles 95 °C 15 s, 57 °C 15 s, 72 °C 30 s; and a melting curve from 72 °C to 95 °C) and analysis were carried out with a StepOne real time-PCR instrument

(Life Technologies) and by the accompanying software (StepOne Software 2.1; Life Technologies).

Growth curve determinations of *B. fragilis* 638R with and without pIP417 (*nimA*). Growth of the *B. fragilis* strain 638R and the same strain with the pIP417 plasmid was observed by densitometry at 600 nm using a UV/VIS spectrophotometer (BioMate; Thermo Electron). Briefly, stationary cultures of the strains in BHI-S broth were diluted 40-fold in parallel in 3 ml aliquots into three glass test tubes of the same media and subsequently incubated anaerobically (BugBox; Ruskinn Technology) at 37 °C. After 5, 10, 15, 20, 25 and 30 h, aliquots in separate tubes were taken out from anaerobiosis, appropriately diluted to obtain OD_{600} readings in the range 0.2–0.6, and their optical densities recorded.

RESULTS AND DISCUSSION

NimA, NimB, NimD and NimE are clearly expressed, and can be visualized by 2DE

In order to demonstrate that *nim* genes are in fact expressed at the protein level, 2DE was performed with cultures of several *B. fragilis* strains that harbour a *nim* gene (Table 1). As a first step, strain *B. fragilis* 638R was analysed because direct comparison was possible between clones without plasmid (*nim* negative) and clones bearing either pIP417 (*nimA*) or pIP421 (*nimD*). The 2D gels of 638R clones, carrying either *nim* gene, were searched for additional protein spots as compared to the *nim*-negative clone. Indeed, additional proteins in the expected molecular mass range of NimA and NimD (18–20 kDa) were readily found (Fig. 1). The proteins were isolated from the gels, analysed by MS and identified as NimA and NimD (Fig. S1). NimA levels, as determined densitometrically with Melanie 2DE-imaging software in 2D gels from four different gel runs at four different time points (four 2D gels in total), ranged from 0.7 to 1.5 % of the total protein visualized ($1.1 \pm$ SEM 0.3 %). Thus, the method enables reproducible measurements of protein levels but does not allow reliable conclusions on differences in abundance below 100 %. NimD levels were found to account for 0.58 % of the total protein visualized. NimB and NimE were also identified in 2D gels from strains BF8 and 388/1, respectively (Table 2). Interestingly, levels of NimB (0.29 % of total protein) and NimE (0.14 % of total protein) were clearly lower than those of NimA and NimD in 638R (Table 2). In the case of *nimB*, this might be attributable not only to a lower rate of transcription and/or translation per se but also to a lower copy number due to its chromosomal location. In contrast, *nimA, nimD* and *nimE* are all episomal (Table 1).

Levels of NimA, NimB and NimE are not further increased upon adaptation to high concentrations of metronidazole

After visualization and measurement of NimA, B, D and E levels in *B. fragilis*, it was assessed whether adaptation to higher concentrations of metronidazole leads to increased levels of Nim proteins. Since Nim proteins have been suggested to act as nitroreductases that reduce metronidazole and other nitroimidazole drugs to nonreactive aminoimidazoles (Carlier *et al.*, 1997), their abundance is expected to be positively correlated with the level of metronidazole resistance. In order to test this, high-level metronidazole resistance (256 mg l^{-1}) was induced in 638R (*nimA*), BF8 (*nimB*) and 388/1 (*nimE*). Interestingly, high-level metronidazole resistance was most easily induced in 388/1, although this strain expresses the lowest level of Nim (Table 2). The strains 638R (*nimA*) and 638R (*nimD*) had previously grown well on plates with 1 μg metronidazole ml^{-1} and somewhat more slowly on plates with 2 μg metronidazole ml^{-1}, whereas 638R (*nim* negative) did not grow at 1 μg metronidazole ml^{-1}, indicating a protective effect of NimA and NimD. High-level metronidazole resistance in 638R (*nim* negative) could not be induced, and attempts to generate clones that were resistant to metronidazole concentrations higher than 16 mg l^{-1} remained unsuccessful. This corroborates previous results (Löfmark *et al.*, 2005) indicating that *nim*-positive strains can be adapted to high metronidazole concentrations much more easily than *nim*-negative strains.

When Nim levels were measured by 2DE in the highly resistant clones, however, no relevant upregulation as compared with the respective parent clones was found (Table 2). In fact, the observed levels of Nim B and E were slightly higher as compared to the unchallenged clones (Table 2), but this is not statistically significant when taking into consideration the observed variance in

Table 1. The *B. fragilis* strains used in this study

MIC was defined as the lowest metronidazole concentration at which further *B. fragilis* growth on WC plates could not be observed.

B. fragilis strain/clone	*nim* gene	Accession nos of Nim proteins	Location of *nim* gene	MIC of metronidazole observed in this study (mg l^{-1})	Reference
638R	None		–	<1	Sebald *et al.* (1990)
638R (*nimA*)	*nimA*	gil435265	pIP417	4	Stubbs *et al.* (2000)
638R (*nimD*)	*nimD*	gil440387	pIP421	4	Stubbs *et al.* (2000)
388/1	*nimE*	gil435261	pBF388C	32	Sóki *et al.* (2004)
BF8	*nimB*	gil70559779	Chromosome	2	Sebald *et al.* (1990)

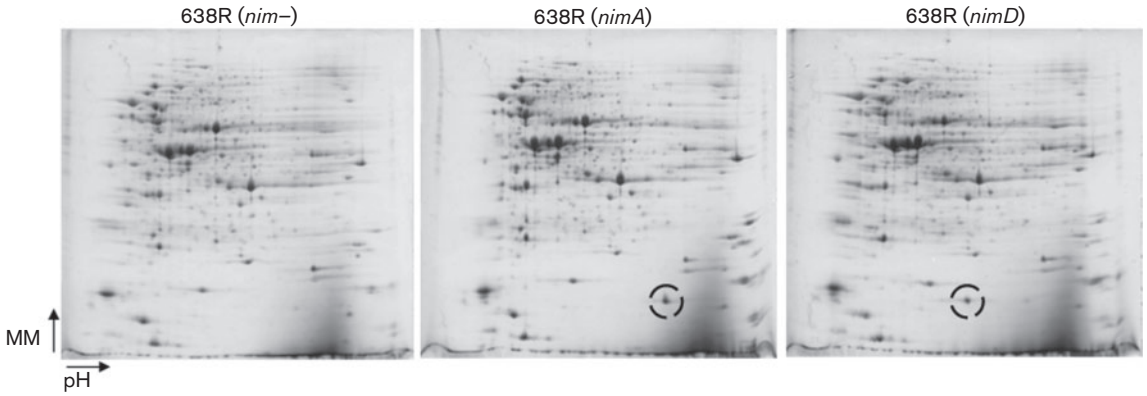

Fig. 1. Representative images of 2D gels (pH range 3–10 nonlinear, 12.5 % polyacrylamide) from strain 638R, either without *nim* gene (left image); with plasmid pIP417 (*nimA*, IS*1186*), *nimA* positive (middle); or with plasmid pIP421 (*nimD*, IS*1169*), *nimD* positive (right). The Nim proteins (encircled) are easily discernible as prominent spots in the lower molecular mass range of the gel. The theoretical molecular masses of NimA and NimD were 20.2 and 18.5 kDa, respectively. The directions of increasing molecular mass (MM) and increasing pH are indicated by arrows.

abundance of NimA in unchallenged 638R (*nimA*) (1.1 ± 0.3 % of total protein visualized). Despite the lack of correlation between Nim levels and the level of metronidazole resistance, copy numbers of plasmid pIP417 (*nimA*) were three- to fourfold higher when 638R (nimA) was grown on plates containing metronidazole than when grown without metronidazole (Fig. 2). Interestingly, copy numbers of the *nimA* gene were not found to be significantly increased in the 638R (*nim*A) clone adapted to 256 mg metronidazole l^{-1} when compared with 638R (*nim*A) routinely grown with 2 mg metronidazole l^{-1} (Fig. 2). Therefore, the reason for the elevated plasmid numbers in 638R (*nimA*) in the presence of metronidazole is unclear. The notion of a selection of clones with higher copy numbers in response to increasing metronidazole concentrations is supported neither by the measured NimA levels (Table 2) nor the insignificant increase of plasmid numbers in the clone adapted to 256 mg metronidazole l^{-1} as compared with the clone adapted to 2 mg metronidazole l^{-1}. However, as metronidazole is mutagenic (Sisson *et al.*, 2000), higher copy numbers of the *nimA* gene might, at least in the first phase of the development of resistance, increase the likelihood of retaining a copy of the gene without deleterious mutations. It is important to note, however, that these speculations apply only to *B. fragilis* strains having a plasmid-borne *nim* gene, and not to those having a chromosomal *nim* gene, e.g. BF8 (Table 1). Nevertheless, the selection of clones with a higher number of chromosomal copies of *nim* genes can not presently be ruled out because copy numbers of *nimB* in BF8 were not determined in this study.

These considerations notwithstanding, no evidence was found for induction of Nim levels upon exposure to increasing doses of metronidazole, nor for a selection of

Table 2. Levels of NimA, NimB and NimE before and after induction of high-level metronidazole resistance (256 mg l^{-1})

Percentages refer to the relative abundance of Nim proteins as compared to total protein visualized by 2DE. The strain carrying the respective *nim* gene is indicated in parentheses.

Level	Nim A (Bf 638R)	Nim B (BF8)	Nim E (388/1)
Original level (%)	0.7	0.29	0.14
Induced level (%)	0.87	0.4	0.18

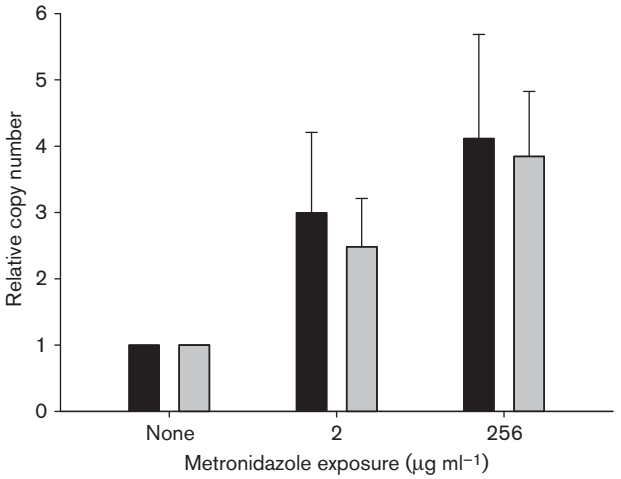

Fig. 2. Relative copy number determination of the *nimA* plasmid (pIP417) depending on metronidazole exposure/adaptation. Error bars indicate SEM with 95 % confidence. Black bars, *nimA*; grey bars, *repA*.

clones expressing higher levels of Nim with increasing level of resistance. In fact, given the comparably low abundance of Nim proteins in the strains BF8 (*nimB* positive) and 388/1 (*nimE* positive), no dependence of the protective effect of Nim proteins on copy number in the cell could be found. It is important to note, however, that NimB and NimE can function differently from NimA and NimD, and that the different genetic background of BF8 and 388/1 allowed lower amounts of Nim to be as effective as the higher amounts of NimA and NimD in 638R.

The protective effect of *nim* genes and an increased propensity of *nim*-positive strains to adapt to high metronidazole concentrations (Löfmark *et al.*, 2005) remains undisputed and is supported by our results presented here. Although our data do not provide any support for the notion of Nim proteins reducing metronidazole, it cannot presently be ruled out that Nim proteins do indeed contribute to metronidazole resistance but require an inducible host factor, either an enzyme or a cofactor, for activity. In this case it would not be the induction of Nim expression that is critical for resistance to be demonstrated, but induction of the hypothetical host factor. The expression level of this host factor could vary from strain to strain, explaining why protective levels of Nim are so variable among different strains (Table 2). Currently, however, there is no indication that such a host factor exists. It is also interesting to note that all our attempts to measure the nitroreductase activity of recombinantly expressed NimA *in vitro* have so far been unsuccessful (data not shown).

NimA is not bound by metronidazole and does not affect the growth rate of *B. fragilis* 638R

Since no correlation of Nim levels and the level of metronidazole resistance was observed, it was hypothesized that Nim proteins might protect sensitive target molecules in *B. fragilis* from low doses of metronidazole by acting as bait and sequestering activated metronidazole. This assumption was mainly based on the observed high levels of Nim (Table 2) and on the propensity of activated metronidazole and other nitroimidazoles to form covalent adducts with defined target proteins (Leitsch *et al.*, 2007, 2009, 2012). As adduct formation with metronidazole can be visualized by 2DE due to a shift in pI of the bound protein to a more alkaline pH (Leitsch *et al.*, 2007, 2009, 2012; Williams *et al.*, 2012), it was speculated that covalent adduct formation of Nim proteins and metronidazole would be discernible on 2D gels. Alternatively, metronidazole treatment may also lead to the degradation of target proteins, as observed with elongation factor 1-γ in *Giardia lamblia* (Leitsch *et al.*, 2012). However, when the levels and integrity of NimA in untreated 638R (*nimA*) and 638R (*nimA*) exposed to 50 μM metronidazole for 2 h were compared, neither a shift nor any degradation of the protein could be observed (Fig. 3a), suggesting that NimA is not a target of metronidazole and, therefore, cannot sequester activated metronidazole. Importantly, adduct formation of metronidazole with several other *B. fragilis* proteins was observed. Of these, one prominent protein spot (2–3 % of all protein visualized) was isolated and analysed by MS. The isolated spot contained four proteins of similar size and pI (Fig. S1): a putative oxidoreductase, a putative D-3-phosphoglycerate dehydrogenase, glutaminase and a putative purine nucleoside phosphorylase. It was not determined which component of the spot was bound by metronidazole, but it is interesting to note that purine nucleoside phosphorylase in the protist parasite *Entamoeba histolytica* was previously identified as a target of metronidazole (Leitsch *et al.*, 2007). Formation of adducts was confirmed in 638R (*nim* negative) with metronidazole and tinidazole (Fig. 3b). Tinidazole forms covalent adducts with the same proteins as metronidazole, but leads to narrower shifts in

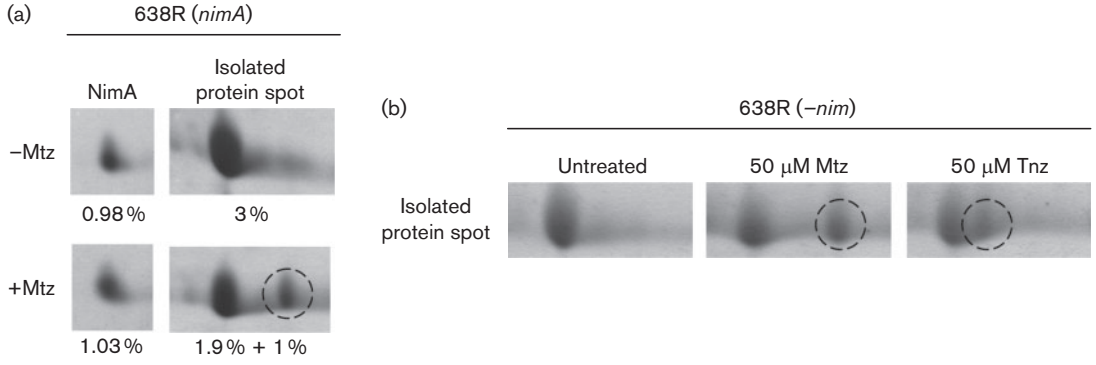

Fig. 3. 2DE analysis of metronidazole-treated 638R. (a) Integrity and relative abundance of NimA and the putative reductase (E1WVQ6_BACF6) were checked on 2D gels after 2 h of incubation of equally divided 638R (*nim*A) stationary phase cultures either without drug or with 50 μM metronidazole. The encircled area indicates the location of the metronidazole adduct of the isolated protein spot. Percentages refer to the relative abundance of the measured proteins as compared to total protein visualized. (b) Validation of metronidazole adduct formation in 638R (*nim* negative). The encircled areas indicate adducts of the isolated protein spot with either metronidazole or tinidazole. Mtz, metronidazole; Tnz, tinidazole.

the pI of bound proteins, presumably due to the different charge of its side chain (Leitsch *et al.*, 2007, 2009, 2012; Williams *et al.*, 2012). Due to this easily discernible difference with regard to the width of pI shift, tinidazole has proven instrumental in distinguishing adduct formation from unspecific changes in proteins upon nitroimidazole treatment. The extent of adduct formation observed in 638R (nim negative) compared well to that in 638R (*nim*A), further arguing against an immediate protective effect of NimA (Fig. 3a, b).

Based on previous results, it was hypothesized that Nim proteins might have an indirect effect on metronidazole sensitivity, e.g. by impeding the metabolism of *B. fragilis*. In fact, metabolically generated reductive power is a prerequisite for the reduction of nitroimidazole drugs (Samuelson, 1999). It was argued that growth rate is a function of metabolic capacity, and growth curves of 638R (*nim* negative) and 638R (*nim*A) were determined in BHI-S broth and in unsupplemented BHI broth, which lack haemin and vitamin K. However, no influence of NimA on growth of 638R (*nim*A) as compared with 638R (*nim* negative) could be found, with the doubling times ($1.39 \pm 0.02\ h^{-1}$ and $1.45 \pm 0.04\ h^{-1}$, respectively) and maximal culture densities (OD_{600} 1.52 ± 0.02 and 1.46 ± 0.03, respectively) of both clones being practically identical. This result argues against any influence of NimA on *B. fragilis* metabolism.

Conclusion

This study represents what is, to the best of our knowledge, the first attempt to quantify Nim levels in *B. fragilis*. No correlation between Nim levels and the level of metronidazole resistance was found, which contrasts with the established notion of Nim proteins inactivating or sequestering metronidazole directly. Further studies on the function of Nim proteins will be necessary to obtain a more reliable notion of their function.

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